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# Synthesis of 2,4-Diamino-6-(thioarylmethyl)pyrido [2,3-*d*]pyrimidines as Dihydrofolate Reductase Inhibitors†

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**Abstract**—Six 2,4-diaminopyrido[2,3-*d*]pyrimidines with a 6-methylthio bridge to an aryl group were synthesized and biologically evaluated as inhibitors of *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) dihydrofolate reductase (DHFR). The syntheses of analogues **3**–**8** were achieved by nucleophilic displacement of 2,4-diamino-6-bromomethylpyrido[2,3-*d*]pyrimidine **14** with various arylthiols. The α-naphthyl analogue **4** showed the highest selectivity ratios of 3.6 and 8.7 against pcDHFR and tgDHFR, respectively, versus rat liver (rl) DHFR. The β-naphthyl analogue **5** exhibited the highest potency within the series with an $IC_{50}$ value against pcDHFR and tgDHFR of 0.17 and 0.09 μM, respectively. Analogue **4** was evaluated for in vitro antimycobacterium activity and was shown to inhibit the growth of *Mycobacterium tuberculosis* $H_{37}Rv$ cells by 58% at a concentration of 6.25 μg/mL.


## Introduction

*Pneumocystis carinii* pneumonia (PCP) and toxoplasmosis are two opportunistic infections commonly diagnosed in immunocompromised patients such as acquired immunodeficiency syndrome and transplant patients.[1] Lipophilic, nonclassical dihydrofolate reductase (DHFR) inhibitors[2] are among the common treatment options against PCP and toxoplasmosis. Classical inhibitors, such as methotrexate (MTX), contain a polar L-glutamic acid moiety in the side chain and therefore require carrier-mediated transport into cells. In nonclassical agents, the polar side chain is replaced with a lipophilic side chain to increase cell permeability. Trimethoprim (TMP)[3] and pyrimethamine[4] (Fig. 1) are two non-classical DHFR inhibitors currently in clinical use. Both are moderately selective towards pathogenic DHFR, however, both are weak inhibitors and require co-administration of sulfonamides to provide synergy and clinical utility.[5] Unfortunately, the combination therapy often results in severe side effects attributed to the sensitivity of some patients to sulfa drugs and results in discontinuation of therapy.[6] Trimetrexate (TMQ)[7,8] and piritrexim (PTX)[9,10] (Fig. 1) are two potent non-classical DHFR inhibitors which lack selectivity toward the pathogenic enzyme and show host toxicity. Leucovorin, the classical folate cofactor 5-formyltetrahydrofolate, must be co-administered with trimetrexate and piritrexim as a host cell rescue agent.[9,10] Leucovorin utilizes the carrier-mediated folate

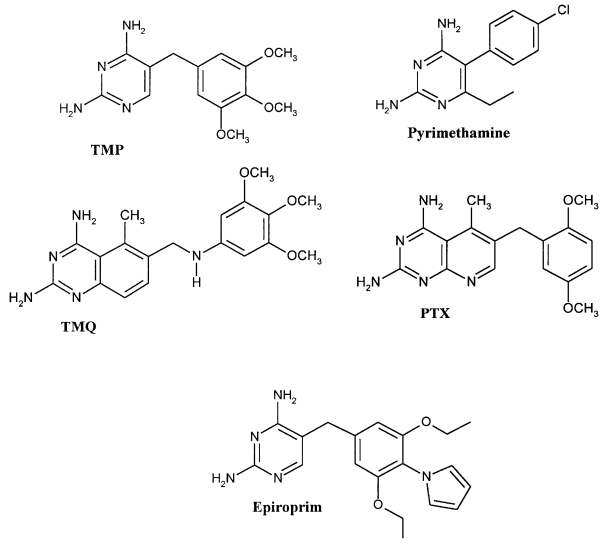


**Figure 1.**

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transport system to enter host cells and circumvent DHFR inhibition. *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) cells lack this transport system. Thus, leucovorin rescue decreases host cell toxicity while the pathogenic cells remain susceptible to the potent inhibition by TMQ and PTX. Drawbacks of the DHFR inhibitor/leucovorin combination therapy include the high cost of leucovorin and the inconsistent effect of leucovorin under all clinical conditions.

Significant strides have been made in the development of analogues that are potent and selective against tgDHFR.[11–13] However, the structural requirements for selectivity against pcDHFR have been more difficult to identify. A *Pneumocystis carinii* selectivity ratio ($IC_{50}$ rat liver (rl) DHFR/$IC_{50}$ pcDHFR)[14] of 11 achieved by TMP has been the standard to which other pcDHFR inhibitors have been compared. Epiroprim[15] (Fig. 1), a trimethoprim analogue, showed a pcDHFR selectivity ratio of 12.8. Piper et al.[12] and Queener et al.[13,16] reported the synthesis and biological activity of 2,4-diamino-6-(phenylthio)methylpteridine (**1**, Fig. 2), a highly selective compound against both pcDHFR and tgDHFR with selectivity ratios (vs rlDHFR) of 25.9 and 319, respectively. The potency, however, against pcDHFR (9.5 μM) and tgDHFR (0.77 μM) was low. In addition, compound **1** lacked potency in cell culture, probaby as a result of a combination of weak DHFR inhibition and inefficient cell penetration.

The selectivities of compound **1** against pcDHFR and tgDHFR were significant, however its lack of potency and in vitro cell culture inhibitory activity prompted us to evaluate the importance of the N5- and N8-nitrogens to biological activity and cell penetration. Calculated log P values show that 5- or 8-deaza analogues of the pteridine **1** have considerably improved lipid solubility, which should translate into better cell penetration.[17,18] Piper et al.,[12] Queener et al.[13] and Gangjee et al.[18–21] have shown that 5-deazapteridine analogues penetrate *Toxoplasma gondii* cells much more proficiently than the corresponding pteridine analogues and are usually more potent. In an attempt to increase potency, cell penetration and in vitro inhibitory activity of **1**, we set out to synthesize the 5-deaza and 8-deaza analogues of **1**.

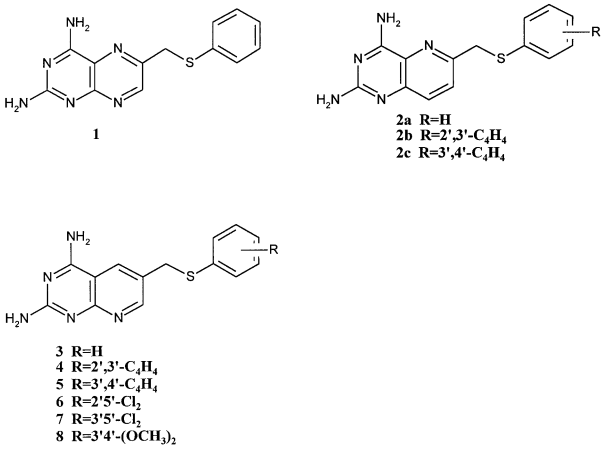
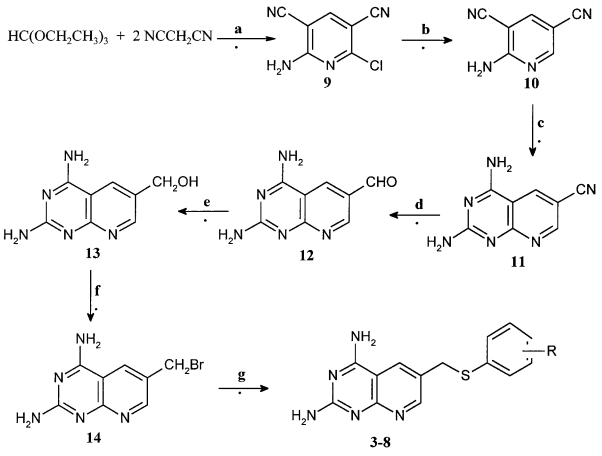



**Figure 2.**

The 2,4-diamino-8-deaza analogues of **1**, compounds **2a–c** (Fig. 2), were reported by Gangjee et al.[22] Compound **2a** was marginally more potent than **1** against pcDHFR and tgDHFR but lost most, if not all, of the selectivity of **1**. This clearly demonstrated that N8 was important for the selectivity of **1**. It now became of interest to determine the contribution of the N5 of **1** to biological activity. This report describes the synthesis of the 2,4-diamino-6-substituted pyrido[2,3-*d*]pyrimidines **3–8** and their inhibitory activities against pcDHFR and tgDHFR and selectivity ratios versus rlDHFR as the reference mammalian enzyme. In addition to analogues containing unsubstituted, methoxy- and chloro-substituted phenyl rings, 1- and 2-naphthyl analogues were also synthesized. The side chains were patterned after compounds that have shown high potencies or selectivites in previous reports.

## Results

The key intermediate in the synthesis of compounds **3–8** was 2,4-diamino-6-bromomethylpyrido[2,3-*d*]pyrimidine (**14**) which was obtained by the modified procedure reported by Piper et al.[23] shown in Scheme 1. Condensation of malononitrile and triethyl orthoformate, followed by treatment with concentrated hydrochloric acid afforded 2-amino-6-chloro-3,5-dicarbonitrilopyridine (**9**). Hydrogenation at 50 psi catalyzed by 5% palladium on barium carbonate gave the reductive dechlorinated product, 2-amino-3,5-dicarbonitrilopyridine (**10**), in 71% yield. Cyclization of **10** with guanidine in sodium ethoxide resulted in 2,4-diamino-6-carbonitrilopyrido[2,3-*d*]pyrimidine (**11**) in 85% yield.




**Scheme 1.** Reagents and conditions: (a) (1) pyridine, reflux, 0.5 h; (2) concentrated hydrochloric acid, 80 °C, 0.5 h; (b) hydrogen at 30 psi, palladium on barium carbonate, 2:1 solution of *N*,*N*-dimenthylformamide/methanol, rt, 5 h; (c) guanidine hydrochoride, sodium ethoxide, reflux, 24 h; (d) 97% formic acid, Raney nickel, reflux, 2 h; (e) Sodium borohydride, methanol, rt, 5 h; (f) hydrogen bromide gas in dioxane, rt, 20 h, or 30% hydrogen bromide in acetic acid, acetic acid, rt, 18 h; (g) potassium carbonate, triethyl amine or sodium hydride, arylthiol, *N*,*N*-dimethylacetamide, rt, 18 h–5 days.

Reduction of the nitrile to the 2,4-diaminopyrido[2,3-*d*]pyrimidne-6-carboxyaldehyde (**12**) was carried out using 97% formic acid and Raney nickel. An attempt to shorten the synthetic route by a one-pot conversion of the nitrile to the alcohol using formic acid, Raney nickel and a longer reaction time resulted in an increase in the intensity of the baseline spots relative to the desired alcohol spot when monitored by thin layer chromatography. Attempts to purify the crude aldehyde by column chromatography resulted in its precipitation in the column due to poor solubility. Thus the crude aldehyde **12** was reduced to 2,4-diamino-6-hydroxymethylpyrido[2,3-*d*]pyrimidine (**13**) with sodium borohydride. The yield for the two-step reduction from the nitrile to the alcohol was 37%. Bromination of the alcohol was achieved using 37% hydrogen bromide in acetic acid. The resulting key intermediate **14** was unstable in air even at low temperatures and thus had to be used immediately without purification. Nucleophilic displacement of the bromide of **14** with the appropriate arylthiol afforded the target compounds **3**–**8** in 6–20% yield. Potassium carbonate, triethyl amine or sodium hydride was used as a base. All three bases were effective at promoting nucleophilic displacement.

The inhibitory activities against pcDHFR, tgDHFR and rlDHFR [defined as the concentration required to inhibit enzyme activity by 50% ($IC_{50}$)] of **3**–**8** are listed in Table 1. Selectivity ratios ($IC_{50}$ against rlDHFR/$IC_{50}$ against pcDHFR or tgDHFR) of the six analogues are also listed in Table 1. Compound **4**, the α-naphthyl analogue, showed the highest selectivity against both pcDHFR and tgDHFR with selectivity ratios of 3.58 and 8.72, respectively. Compound **4** was also tested in a tuberculosis antimicrobial screening assay directed by the Southern Research Institute.[25] At a concentration of 6.25 μg/mL, compound **4** inhibited the growth of *Mycobacterium tuberculosis* $H_{37}Rv$ cells by 58%.

**Table 1.** Inhibition concentrations ($IC_{50}$, in μM) of dihydrofolate reductase from *P. carinii*, *T. gondii* and rat liver and selectivity ratios[a]

| | R | pcDHFR | rlDHFR | rl/pc | tgDHFR | rl/tg |
|---|---|---|---|---|---|---|
| **1** | | 9.5 | 246 | 25.9 | 0.77 | 319 |
| **2a** | | 2.0′ | 0.52 | 0.26 | 0.13 | 4.0 |
| **2b** | | 0.47 | 0.16 | 0.34 | 0.049 | 3.3 |
| **2c** | | 0.38 | 0.086 | 0.23 | 0.048 | 1.8 |
| **3** | H | 1.3 | 1.9 | 1.46 | 0.47 | 4.04 |
| **4** | 2′,3′-$C_4H_4$ | 0.95 | 3.4 | 3.58 | 0.39 | 8.72 |
| **5** | 3′,4′-$C_4H_4$ | 0.17 | 0.22 | 1.29 | 0.09 | 2.44 |
| **6** | 2′,5′-$Cl_2$ | 5.9 | 2.5 | 0.42 | 2 | 1.25 |
| **7** | 3′,5′-$Cl_2$ | 11 | 38 | 3.45 | 6.2 | 6.13 |
| **8** | 3′,4′-$(OCH_3)_2$ | 2.2 | 4 | 1.82 | 1.1 | 3.64 |
| TMP | | 12 | 133 | 11.1 | 2.7 | 49 |
| Pyrimethamine | | 3.7 | 2.3 | 0.62 | 0.39 | 5.9 |
| TMQ | | 0.042 | 0.003 | 0.07 | 0.010 | 0.3 |
| PTX | | 0.031 | 0.0015 | 0.048 | 0.017 | 0.088 |
| Epiroprim | | 2.6 | 33.2 | 12.8 | 0.48 | 70.6 |

[a]These assays were carried out at 37 °C, under conditions of substrate (90 μM dihydrofolic acid) and cofactor (119 μM nicotinamide diphosphate) in the presence of 150 mM potassium chloride and 2-mercaptoethanol (8.9 mM), in a sodium phosphate buffer (pH 7.4).[13]

## Discussion

Comparison of the $IC_{50}$ values of **3** and **1** suggests that, with identical side chains, the 2,4-diaminopyrido[2,3-*d*]pyrimidine ring structure imparts greater inhibitory effects against DHFR than does the 2,4-diaminopteridine ring structure. Analogue **3** was 7-, 129- and 1.6 fold more potent than **1** against pcDHFR, rlDHFR and tgDHFR, respectively. While the increase in inhibitory activities against the pathogenic DHFR of **3** compared to **1** were appreciable, the increase in inhibitory activity of **3** against rlDHFR was much greater, thereby abolishing the selectivity compared to **1**. Thus, the replacement of the N5 with a CH increased potency but decreased selectivity.

Compared to the N8-deaza analogue **2a**, compound **3** was slightly more potent against pcDHFR and less potent against rlDHFR. This afforded a six-fold increase in pcDHFR selectivity. Thus, while the N5-deaza analogue afforded greater selectivity than the N8-deaza analogue, the isosteric replacement of either the N5 or the N8 of the pteridine ring structure with a carbon atom results in analogues with decreased selectivity compared to compound **1.** However, the replacement of N5 or N8 with a carbon atom results in analogues with increased potency compared to **1**.

The α-naphthyl analogue **4** was equipotent with **3** against pcDHFR and tgDHFR. A two-fold decrease in potency against rlDHFR afforded a two-fold increase in selectivity against pcDHFR and tgDHFR for **4** compared to **3**. Analogue **4**, with selectivity ratios of 3.6 and 8.7 for pcDHFR and tgDHFR, respectively, exhibited the highest degree of selectivity of the series against both pathogenic enzymes. The β-naphthyl analogue **5** was the most potent of the series against both pathogenic enzymes with $IC_{50}$ values of 0.17 and 0.09 μM against pcDHFR and tgDHFR, respectively. A comparison of the 1- and 2-naphthyl substituted analogues **4** and **5** with the corresponding analogues **2b** and **2c** of the pyrido[3,2-*d*]pyrimidines indicates that the selectivities of **4** and **5** are higher. The selectivity ratio of **4** is 10-fold higher than that of **2b** against pcDHFR. However, the potencies of the naphthyl substituted pyrido[2,3-*d*]pyrimidine analogues are generally lower compared to the corresponding pyrido[3,2-*d*]pyrimidine analogues (with the exception of **5** against pcDHFR.)

Analogues of **3** with electron-donating or electron-withdrawing substituents on the phenyl ring result in decreased potency against pcDHFR and tgDHFR, and increased potency against rlDHFR compared to **3** with a consequent loss of selectivity. In the pyrido[2,3-*d*]pyrimidine series of this study, the dichlorophenyl analogues **6** and **7**, as well as the dimethoxyphenyl analogue **8** similarly showed a loss in activity against pcDHFR, tgDHFR and rlDHFR. The differences in selectivities of **6**–**8** varied with respect to **3**. Compound **6**, the 2′,5′-dichloro analogue, was less selective against both pcDHFR and tgDHFR compared to compound **3**, while compound **7**, the 3′,5′-dichloro analogue, was more selective against the two pathogenic DHFR

compared to **3**. This suggests that although chloro substituents on the phenyl ring appear to unfavorably affect potency, their effect on selectivity ratios is dependent on the position of the substitutents in the phenyl ring.

In summary, the combination of the 2,4-diaminopyrido[2,3-*d*]pyrimidine ring system and the thiolarylmethyl moiety of compounds **3**–**8** resulted in increased potencies against pcDHFR for all analogues reported in this series (with the exception of **7**) compared to **1**. Against tgDHFR, analogues **3**–**5** also exhibited increased potency compared to **1**. The dichloro and dimethoxy substitutions on the phenyl ring proved detrimental to tgDHFR inhibitory activity compared to **1**. However, the substantial increase in potency against rlDHFR in analogues **3**–**8** was the major contributing factor to the loss of selectivity compared to **1**.

Under the direction of the National Institute of Allergy and Infectious Diseases (NIAID), the Tuberculosis Antimicrobial Acquisition and Coordinating Facility at Southern Research Institute selected compound **4** for an in vitro evaluation of antimycobacterial activity. For primary screening, *Mycobacterium tuberculosis* $H_{37}Rv$ cells (ATCC 27294) were treated with a drug concentration of 6.25 mg/mL. Compound **4** inhibited the growth of ATCC 27294 cells by 58% at this concentration.

## Experimental

Thin-layer chromatography was performed on silica gel plates with fluorescent indicator purchased from Aldrich Chemical Company, Milwaukee, WI. UV light at 254 and 365 nm was used for visualization. Column chromatography was carried out using silica gel, 200–400 mesh, purchased from Aldrich Chemical Company. High resolution mass spectral analyses were obtained on a VG 70G or micromass AUTOSPEC double focusing mass spectrometer. Samples were introduced by direct insertion probe. $^1$H NMR spectra were recorded on a Brucker WH-300 (300 MHz) instrument. The chemical shift (δ) values are expressed in part per million (ppm) relative to tetramethylsilane (TMS) as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad peak, exch = exchangeable by addition of $D_2O$. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. Elemental compositions were within ± 0.4% of the calculated value. Fractional moles of solvents in the analytical samples frequently found in antifolates could not be prevented in spite of vigorous drying in vacuo and were confirmed, where possible, by their presence in the NMR spectrum. Melting points were determined on a MEL-TEMP II melting point apparatus with a Fluke 51 K/J electronic thermometer and are uncorrected.

**General procedure for the synthesis of compounds 3–8. (Procedure A).** To a cooled solution (0–5 °C) of the appropriate arylthiol dissolved in *N*,*N*-dimethyl acetamide or *N*,*N*-dimethyl formamide was added sodium hydride, triethyl amine or potassium carbonate. The solution was stirred under nitrogen for 15 min before intermediate **14** was added. The reaction was allowed to warm to room temperature and stirred for 24 h under nitrogen. The desired product precipitated out of solution. The yellow solid was filtered and washed with water, ethanol and ether. Thin layer chromatographic analyses were performed in two solvent systems (Solvent A: 3:1, chloroform/methanol and Solvent B: 5:1:3 drops, chloroform/methanol/ammonium hydroxide). In the preparation of analogues **3**–**5**, the precipitate was analytically pure and required no further purification. In the preparation of analogues **6**–**8**, the crude precipitate was redissolved in warm *N*,*N*-dimethyl formamide and 1 g of silica gel added to this solution and the solvent was evaporated to afford a plug of silica gel for column chromatographic purification. The crude product silica gel plug was loaded onto a silica gel column and eluted with solvent prepared in a 5:1 ratio of chloroform/methanol. Fractions shown by thin layer chromatography to contain pure product were pooled and evaporated to afford a light yellow solid. The solid was washed with water, ethanol and ether. All solids were dried in vacuum with the aid of phosphorous pentoxide.

**2,4-Diamino-6-[(phenylthio)methyl]pyrido[2,3-*d*]pyrimidine (3).** Compound **3** was synthesized from intermediate **14** (0.25 g, 1.0 mmol), phenylthiol (0.12 mL, 1.2 mmol) and potassium carbonate (1.0 g) in 15 mL of *N*,*N*-dimethylacetamide using Procedure A to afford a light yellow solid (0.022 g, 8%): mp > 189 °C dec; TLC $R_f$ 0.33 in solvent A; $^1$H NMR (DMSO-$d_6$) δ 4.28 (s, 2H, $CH_2$), 7.19 (m, 2H, $ArH_5$), 7.32 (m, 3H, $ArH_5$, 2H, $NH_2$, exch), 8.40 (br s, 2H, $NH_2$, exch), 8.52 (s, 1H, 5-H), 8.62 (s, 1H, 7-H). HR-MS: $C_{14}H_{13}N_5S$: calcd. mass 283.089167, found mass 283.088811.

**2,4-Diamino-6-[(1-naphthylthiol)methyl]pyrido[2,3-*d*]pyrimidine (4).** Compound **4** was synthesized from intermediate **14** (0.12 g, 0.49 mmol), 1-naphthalenethiol (0.088 g, 0.55 mmol) and sodium hydride, 80% dispersion in mineral oil (0.016 g) in 15 mL of *N*,*N*-dimethylacetamide using Procedure A to afford a yellow solid (0.028 g, 17%): mp > 278 °C dec; TLC $R_f$ 0.41 in solvent B; $^1$H NMR (DMSO-$d_6$) δ 4.35 (s, 2H, $CH_2$), 6.58 (s, 2H, $NH_2$, exch), 7.46 (m, 3H, $ArH_7$), 7.78 (m, 4H, $ArH_7$, 2H, $NH_2$, exch), 8.44 (s, 1H, 5-H), 8.61 (s, 1 H, 7-H). HR-MS: $C_{18}H_{15}N_5S$: calcd. mass 333.104817, found mass 333.105846.

**2,4-Diamino-6-[(2-naphthylthiol)methyl]pyrido[2,3-*d*]pyrimidine (5).** Compound **5** was synthesized from intermediate **14** (0.12 g, 0.49 mmol), 2-naphthalenethiol (0.088 g, 0.55 mmol) and sodium hydride, 80% dispersion in mineral oil (0.016 g) in 15 mL of *N*,*N*-dimethylacetamide using Procedure A to afford a yellow solid (0.033 g, 20%): mp > 236 °C dec; TLC $R_f$ 0.43 in solvent B; $^1$H NMR (DMSO-$d_6$) δ 4.27 (s, 2H, $CH_2$), 6.31 (s, 2H, $NH_2$, exch), 7.46 (m, 1H, $ArH_7$), 7.56 (m, 3H, $ArH_7$, 2H, $NH_2$, exch), 8.79 (d, 1H, $ArH_7$), 8.91 (d, 1H, $ArH_7$), 8.22 (d, 1H, $ArH_7$), 8.33 (s, 1H, 5-H), 8.50 (s, 1 H, 7-H). HR-MS: $C_{18}H_{15}N_5S$: calcd. mass 333.104817, found mass 333.105804.

**2,4-Diamino-6-[(2′,5′-dichlorophenylthio)methyl]pyrido[2,3-*d*]pyrimidine (6).** Compound **6** was synthesized from intermediate **14** (0.25 g, 1.0 mmol), 2,5-dichlorophenylthiol (0.27 g, 1.5 mmol) and triethyl amine (0.5 mL) in 15 mL of *N*,*N*-dimethylformamide using Procedure A to afford a yellow solid (0.048 g, 14%): mp $\geq$246 °C dec; TLC $R_f$ 0.57 in solvent A; $^1$H NMR (DMSO-$d_6$) δ 4.39 (s, 2H, $CH_2$), 6.68 (s, 2H, $NH_2$, exch), 7.29 (dd, 1H, $ArH_3$), 7.50 (m, 2H, $ArH_3$), 7.84 (s, 2H, $NH_2$, exch), 8.47 (d, 1H, 5-H), 8.69 (d, 1 H, 7-H). HR-MS: $C_{14}H_{11}N_5SCl_2$: calcd. mass 351.011223, found mass 351.009459.

**2,4-Diamino-6-[(3′,5′-dichlorophenylthio)methyl]pyrido[2,3-*d*]pyrimidine (7).** Compound **7** was synthesized from intermediate **14** (0.25 g, 1.0 mmol), 3,5-dichlorophenylthiol (0.27 g, 1.5 mmol) and sodium hydride, 80% dispersion in mineral oil (0.10 g) in 25 mL of *N*,*N*-dimethylformamide using Procedure A to afford a yellow solid (0.039 g, 11%): mp $\geq$198 °C dec; $^1$H NMR (DMSO-$d_6$) δ 4.37 (s, 2H, $CH_2$), 6.44 (s, 2H, $NH_2$, exch), 7.49 (s, 3H, $ArH_3$), 7.59 (s, 2H, $NH_2$, exch), 8.39 (s, 1H, 5-H), 8.62 (d, 1 H, 7-H). HR-MS: $C_{14}H_{11}N_5SCl_2$: calcd. mass 351.011223, found mass 351.009850.

**2,4-Diamino-6-[(3′,4′-dimethoxyphenylthio)methyl]pyrido[2,3-*d*]pyrimidine (8).** Compound **8** was synthesized from intermediate **14** (0.25 g, 1.0 mmol), 3,4-dimethoxyphenylthiol (0.18 g, 1.06 mmol) and sodium hydride, 80% dispersion in mineral oil (0.03 g) in 15 mL of *N*,*N*-dimethylformamide using Procedure A to afford a yellow solid (0.021 g, 6%): mp >247 °C dec; TLC $R_f$ 0.30 in solvent A; $^1$H NMR (DMSO-$d_6$) δ 3.67 (s, 3H, $OCH_3$), 3.74 (s, 3H, $OCH_3$), 4.27 (s, 2H, $CH_2$), 6.74 (m, 1H, $ArH_3$), 6.82 (d, 1H, $ArH_3$), 6.95 (d, 1H, $ArH_3$), 8.67 (d, 1H, 5-H), 8.73 (s, 1 H, 7-H) 8.99 (br d, 4H, $NH_2$, $NH_2$, exch). HR-MS: $C_{16}H_{17}N_5SO_2$: calcd. mass 343.110297, found mass 343.109170.

**Dihydrofolate reductase (DHFR) assay was preformed as previously described.**[13] The spectrophotometric assay for DHFR was modified to optimize for temperature, substrate concentration, and cofactor concentration for each enzyme form assayed.[13] The standard assay contained sodium phosphate buffer pH 7.4 (40.7 mM); 2-mercaptoethanol (8.9 mM); NADPH (0.117 mM); 1 to 3.7 International Units of enzyme activity (1 International Unit = 0.005 OD units/min); and dihydrofolic acid (0.092 mM). KCl(150 mM) was included in the assay for tgDHFR and rlDHFR, because it stimulated the enzymes 1.4- and 2.63-fold, respectively. KCl was omitted from assays of pcDHFR because no stimulation was produced with high salt. The first three reagents were combined in a disposable cuvet and brought to 37 °C. Drug dilutions were added at this stage. The enzyme was added 30 s before the reaction was initiated with dihydrofolic acid. The reaction was followed for 5 min with continuous recording. Activity under these conditions of assay was linear with enzyme concentration over at least a four-fold range. Background activity measured with no added dihydrofolic acid was zero with the enzyme obtained from cultured *T. gondii* and near zero for other forms of DHFR. All DHFR inhibitors were tested against rlDHFR as well as against pathogen DHFR to allow assessment of selectivity.

**Determination of $IC_{50}$ values.** DHFR was assayed without inhibitor and with a series of concentrations of inhibitors to produce 10 to 90% inhibition. At least three concentrations were required for calculation. Semilogarithmic plots of the data yielded normal sigmoidal curves for most inhibitors. The data were converted from percent inhibition to probit values, which were plotted versus the log of the drug concentration. The resultant straight lines were analyzed by least-squares linear regression. The 50% inhibitory concentration ($IC_{50}$) is the concentration at which the probit value is 5.0.

**Source of *T. gondii*.** A frozen sample of the RH strain of *T. gondii* was obtained from the Centers for Disease Control, Atlanta, GA, and inoculated intraperitoneally into female ICR mice (Harlan Industries, Indianapolis). Peritoneal exudate was collected 7 days later and found to contain numerous *T. gondii* organisms, as well as many host cells, some of which were infected with *T. gondii*. Approximately $2\times10^6$ *T. gondii* were inoculated intraperitoneally into new host mice. The organisms grew more rapidly on second passage in mice and were harvested within 4 days after inoculation. Harvests were scaled up to 20 mice and peritoneal exudate was pooled, centrifuged, and the organisms were resuspended in RPMI medium containing 10% fetal calf serum. Frozen stocks were prepared by adding 5% DMSO to the medium, and freezing slowly over 8–15 h. Stocks were stored in liquid nitrogen.

A clinical isolate of *T. gondii* was obtained from the Department of Pathology, Indiana University School of Medicine. This strain was handled as described above for the RH strain and stocks were prepared in liquid nitrogen. The clinical isolate displayed kinetics of growth in culture that are more advantageous for production of enzymes and is now a standard source of DHFR from *T. gondii*. The material was maintained as described in the previous paragraph for the RH strain.

**Culture of *T. gondii* for enzyme production.** *T. gondii* was grown on a chinese hamster ovary cell line that lacks DHFR (American Type Culture Collection, 3952 CL, CHO/dhfr-).[13a] Cells were maintained in Iscove's Modified Eagle's Medium with 10% fetal calf serum, 1% penicillin/ streptomycin, 100 mM hypoxanthine, and 10 mM thymidine. An inoculum of approximately $10^7$ organisms was added to each 75 $cm^2$ tissue culture flask containing the monolayer of cells. Within 6–8 days, $4\times10^8$ organisms were harvested from each flask.

**Preparation of enzymes from *T. gondii*.** When harvested from tissue culture, *T. gondii* organisms were minimally contaminated with mammalian host cells, which in any case should contain no DHFR activity. To confirm this property, uninoculated monolayer cultures from three flasks were combined, sonicated, and the 100,000*g*

supernate was assayed for DHFR; no DHFR activity was detected. Organisms from culture were washed in phosphate-buffered saline containing 10 mM citrate, and resuspended in 50 mM phosphate buffer (pH 7.0) containing leupeptin (20 μg/mL), phenylmethylsulfonyl fluoride (9 μg/mL), soybean trypsin inhibitor (50 μg/mL), aprotinin (50 μg/mL), and 20 mM 2-mercaptoethanol. This buffer released the cytoplasmic contents of *T.* gondii. The suspension was centrifuged at 100,000*g*. The 100,000*g* supernates from *T. gondii* prepared in culture contained both DHFR and dihydropteroate synthase (DHPS) activity. The presence of DHPS, which is not found in mammalian cells, and the absence of DHFR in the specific host cell line used for culture supports the conclusion that the DHFR activity measured arose from *T. gondii*. The preparation was stored in liquid nitrogen without appreciable loss of activity. The yield of DHFR from *T. gondii* cultures was approximately 40 IU per flask.

DHFR from cultured *T. gondii* was tested with known inhibitors of the enzyme. $IC_{50}$ values calculated for methotrexate and pyrimethamine were 0.014 and 0.24 μM, respectively; the $IC_{50}$ values agreed closely with the reported values of 0.021 and 0.76 μM.[24] The $IC_{50}$ value for pyrimethamine with DHFR prepared from *T. gondii* harvested from mice was 0.39 μM, a value in close agreement with the $IC_{50}$ for the enzyme from cultured *T. gondii*. The $IC_{50}$ value for TMP was 1.8, 2.9, and 3.5 μM in three independent trials with enzyme from cultured *T. gondii,* but was reported by others to be 14.5 μM for the enzyme prepared from organisms harvested from mice.[24]

Kinetics for cofactor and substrate were determined for DHFR from rat liver and *T. gondii*. Both forms of DHFR displayed kinetics for dihydrofolic acid under conditions of assay that differed from the Michaelis–Menten model.[25] Curves suggested substrate inhibition at concentrations above that used in the standard assay. The kinetic parameters of rat liver DHFR and *T. gondii* DHFR were similar, with half maximal rates at 11 to 13 μM dihydrofolic acid, respectively. Others have also suggested that substrate kinetics of the two forms of DHFR are similar.[26] Kinetics for NADPH followed the Michaelis–Menten model and yielded linear double reciprocal plots; $K_m$ values of 11 and 23 μM were determined for DHFR from *T. gondii* and rat liver, respectively. Others reported a $K_m$ of 6.7 μM for NADPH with DHFR from *T. gondii*.[26] These kinetic studies and studies using known inhibitors of tgDHFR confirmed that the enzyme from cultured *T. gondii* was similar to preparations previously reported. Based upon these studies with DHFR, cultured *T. gondii* were used as a standard source for enzyme preparations.

**Isolation of pcDHFR.** Recombinant pcDHFR was produced for enzyme assays. The gene sequence was identical to that previously reported.[27] The expression system used pET8C, which employs the T7 RNA polymerase.[28] Host *E. coli* containing the appropriate plasmid construction was grown in Luria broth culture with 75 μg/mL kanamycin at 37 °C on a rotary shaker. The culture was transferred to fresh medium and $OD_{590}$ was monitored. When the $OD_{590}$ reaches 0.4, the culture was shifted to 42 °C for 30 min to induce the gene for T7 RNA polymerase. Rifampin 200 μg/mL was added to suppress *E. coli* RNA polymerase. After 30 min, the culture was shifted back to 37 °C for 90 min. Cells were harvested by centrifugation, washed, and suspended in appropriate buffer containing protease inhibitors and 2-mercaptoethanol as described above. Bacterial cells were ruptured by sonication. The 100,000*g* supernate containing recombinant pcDHFR has been the standard enzyme used in the screen under contract NO1-Al-35171. Studies with [$^{35}$S]methionine incorporation have shown this preparation to contain predominately one strong band on autoradiography at a molecular weight corresponding to DHFR.

**In vitro evaluation of antimycobacterial activity.** Primary screening is conducted at 6.25 mg/mL against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC[29] 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay. Compounds exhibiting fluorescence are tested in the BACTEC 460 radiometric system.

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